

PATENT Customer No. 22, 852 Attomey Docket No. 08888.0517

#### IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| In re  | Application of:                                                         | ) |                          |
|--------|-------------------------------------------------------------------------|---|--------------------------|
| Frai   | ncis BLANCHE et al.                                                     | ý |                          |
| Applio | cation No.: 09/970,663                                                  | ) | Group Art Unit: 1635     |
| Filed: | October 5, 2001                                                         | ) | Examiner. Brian Whiteman |
| For:   | COMPOSITION FOR THE PRESERVATION OF INFECTIOUS RECOMBINANT ADENOVIRUSES | ) |                          |
| P.O.   | missioner for Patents<br>Box 1450<br>andria, VA 22313-1450              |   |                          |
| Sir    |                                                                         |   |                          |

#### **DECLARATION UNDER 37 C.F.R. § 1.131**

We, Francis Blanche and Shian-Jiun Shih, state that we are the named applicants of the above-identified application and that we are co-inventors of the subject matter described and claimed therein. Prior to November 16, 1998, we, the co-inventors, had completed in France the invention as described and claimed in the above-identified application as evidenced by the following:

1. Exhibit A: Laboratory Notebook Pages 51-55 and 176 (A1-A6) of Francis Blanche, showing, a composition comprising adenoviral particles and a glycerol buffer solution at pH 8.4, wherein the buffer solution does not contain added divalent metal cations or alkali metal cations. See pages 52-53 (A2-A3), formulation #2, for example, comprises Tris/HCl and 10% glycerol at pH 8.4 (hereinafter referred to as "formulation #2".) The addition of adjuvants, such as sucrose or Tween20 is shown, for example, at page 176, formulations C and D. Formulation #2 is shown to be

Application No. O9/970,663 Attorney Docket No. O3806.0517

useful for preserving adenoviruses. See page 55 (A5), stable viral titer after 15 days of storage in formulation #2. Some compositions were tested for stability after –20°C or 4°C storage, indicating that the –20°C frozen viral compositions were thawed to test viability. See page 176 (A6), last three lines from the bottom.

- 2. The present specification at page 17, first formulation in the Table, shows a formulation identical to formulation #2 of Exhibit A;
- 3. Example 3 of the present specification, at pages 18-19, shows that a formulation identical to formulation #2 of Exhibit A has a stable viral titer after 15 days of storage, similar to the 15-day storage stability of formulation #2 shown on page 55 (A5) of Exhibit A.

While the dates have been redacted, the undersigned testify that all experiments described herein were conducted before November 16, 1998.

We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

| Dated: 25 Mynt, 2003 | By: Francis Blanche Francis Blanche |
|----------------------|-------------------------------------|
|                      |                                     |
| Dated:, 2003         | By:<br>Shian-Jiun Shih              |

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# ESSAIS FORMULATIONS STABILITE

BUT : Observer la stabilité ou la précipitation éventuelle du virus Y28 dans différentes formulations.

#### MATERIEL VIRAL ETUDIE:

Solution virale Y28 produite en Cell Cube à l'échelle 8 Mer par l'équipe JF Chaubard et purifiée par chromatographie échangeuse d'anions, conservée dans le Tris 20mM pH8, MgCl<sub>2</sub> 1mM, NaCl 500mM et glycerol 10%. Le virus purifié titre 3,94.10<sup>11</sup> pv/ml.

#### PREPARATION DES DIFFERENTS TAMPONS ETUDIES :

#### 1. Solutions mères :

|          | SOLUTIONS MERES:             | PREPARATIONS:                                                                                        |
|----------|------------------------------|------------------------------------------------------------------------------------------------------|
|          |                              |                                                                                                      |
| · ·      | Tris / HCl pH 8,4 à 500mM    | 10,07g Tris base + 6,60g Tris/Hcl dans 250ml eau PPI<br>(Tris base ref: T8524 et Tris HCL ref:T7149) |
|          | Sucrose à 50g/100ml          | 250g de sucrose dans 500ml d'eau PPl                                                                 |
|          | Sucrose a Jugitonia          |                                                                                                      |
|          | NaCl 5M                      | Sigma - Aldrich ref.\$150                                                                            |
| )        | MgCl <sub>2</sub> 1M         | Sigma - Aldrich ref.M1028                                                                            |
|          |                              |                                                                                                      |
| E :      | Glycerol                     | Sigma - Aldrich ref.G5516                                                                            |
| F        | D-Mannitol                   | Sigma - Aldrich ref.M9647                                                                            |
| G        | Tween 20                     | Sigma - Aldrich ref.P8074                                                                            |
| <u> </u> |                              |                                                                                                      |
| H        | Tampon borate pH 7.4 100mM   | Acide borique 100mM + NaOH 0.1N                                                                      |
|          | Tampon phosphate pH 7.4 10mM | 130mg KH <sub>2</sub> PO <sub>4</sub> + 705mg K <sub>2</sub> HPO <sub>4</sub> dans 500ml eau Ph      |

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ESSAI N° \_\_\_\_\_

A-2

SI

2. Formulations:

|           | •      |              |             |                          |                                        |                                        |               |                                        |              |                                                    |
|-----------|--------|--------------|-------------|--------------------------|----------------------------------------|----------------------------------------|---------------|----------------------------------------|--------------|----------------------------------------------------|
| ·         |        |              |             | <u>S</u>                 | OLUTIONS                               | MERES                                  |               |                                        |              |                                                    |
| 7         |        |              |             | _                        | •                                      |                                        | <del></del>   |                                        | -            | Eau PPI                                            |
|           |        | В            | С           | · D                      | E                                      | F                                      | G             | H                                      | <del></del>  | Lau FF1.                                           |
|           | A      | <del></del>  |             |                          |                                        |                                        |               |                                        |              |                                                    |
| SSAIS:    |        |              |             |                          |                                        |                                        |               |                                        |              | 400                                                |
|           |        |              |             |                          |                                        |                                        |               |                                        |              | qsp 500m                                           |
| <u>1</u>  | 20ml   |              | <del></del> | <u> </u>                 |                                        |                                        |               |                                        |              |                                                    |
|           |        |              |             | T                        | + 50ml                                 |                                        |               |                                        |              | qsp 500m                                           |
| 2         | 20ml   |              |             | l                        |                                        |                                        |               |                                        |              |                                                    |
|           |        |              |             | 0,5ml                    |                                        |                                        |               |                                        |              | qsp 500m                                           |
| 3         | 20ml   | 50ml         | L           | 0,5111                   | L                                      |                                        |               |                                        |              |                                                    |
|           |        | <u> </u>     |             | <del></del>              |                                        |                                        |               |                                        | لمنسيا       | qsp 500m                                           |
| 4         | 20ml   | 50ml         | <u> </u>    |                          |                                        |                                        |               |                                        |              | <u>.</u>                                           |
|           |        |              | ,           | 1 06 1                   | · ·                                    | 25g                                    |               |                                        |              | qsp 500n                                           |
| 5         | 20ml . | 50ml         | <u>l</u>    | 0.5ml                    | 1                                      |                                        |               | · · · · · · · · · · · · · · · · · · ·  |              |                                                    |
|           |        | <u> </u>     |             |                          |                                        | 25g                                    | T             |                                        |              | qsp.500m                                           |
| 6         | 20ml   | 50ml         | 15ml        | i 0.5ml                  | نـــــــــــــــــــــــــــــــــــ   | 1 235                                  | <del></del>   |                                        |              | 119                                                |
|           |        | •            | •           |                          | <del></del>                            | 100                                    | 0,5ml         | · ·                                    |              | qsp 5001                                           |
| 7         | 20ml   | 50ml         | <u> </u>    | <u> </u>                 |                                        | 1                                      | . 0,544       |                                        |              |                                                    |
|           | · .    |              |             |                          | <del>,</del>                           |                                        | 0.5ml         | 1                                      |              | qsp 500r                                           |
| 8         | 20ml   | 50ml         |             | 0,5ml                    | ــــــــــــــــــــــــــــــــــــــ | 1                                      | 1 0.5111      |                                        | <del></del>  |                                                    |
|           |        |              | ·           |                          |                                        |                                        |               | 50ml                                   |              | qsp 5001                                           |
| 9         | T -    | 50ml         |             | 0,5ml                    |                                        | <u> </u>                               | <del> ا</del> | 301111                                 |              |                                                    |
|           |        | <del> </del> | · · ·       |                          |                                        |                                        |               | T                                      | 500ml        | T .                                                |
| 10        | $\top$ | T            | T           |                          | + 50ml                                 | ــــــــــــــــــــــــــــــــــــــ |               | ــــــــــــــــــــــــــــــــــــــ | 1 20000      | <del></del>                                        |
| 10        |        |              |             |                          |                                        |                                        |               |                                        | -1-          |                                                    |
|           |        |              | Solutiony   | rale obtenu              | e au 2eme r                            | incage lor                             | s de la diafi | itration Dir                           | <u> 415.</u> | •                                                  |
| <u>]]</u> | 1      |              | Titre =     | 2.88.10 <sup>11</sup> pv | ml dans D                              | PBS/ NaC                               | 1 150mM /g    | JVCCTOL 107                            | <u>′°</u>    | <del>` · · · · · · · · · · · · · · · · · · ·</del> |
|           | l      |              |             |                          |                                        | ξ.                                     |               |                                        |              |                                                    |

#### 3 Résumé des formulations étudiées :

Voir tableau ci-après.

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Y28 CELL CUBE 8MER
ESSAIS DE FORMULATIONS

PCC (FIGURE)

in a charte

W. W.

#### MATERIELS UTILISES:

- →10 PD 10 pour la diafiltration équilibrées avec 5 x 5ml de tampon étudié.
- →Ultrafree 15 ml avec membrane Biomax 100 Kd (Millipore) (2x pour chaque essai).
- →Centrifugeuse réglée à 1500 tr/mn.

#### MISE EN OEUVRE :

| OPERATIONS:                        | POUR CHAQUE ESSAl:                                                                                                                                                                                                                                  |
|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| IAFILTRATION:                      | 10 PD10 x 2,5ml de solution virale Y28 à 3,94.10 <sup>11</sup> pv/ml. Elution par 10PD10 x 3,5ml du tampon étudié.                                                                                                                                  |
| ONCENTRATION:                      | 2 Ultrafree 15ml 100Kd remplie à 15ml puis rechargés avec 2,5ml de solution virale diafiltrée.  Soit 17,5ml concentrés à 500µl (x2).  (soit une concentration à ≈ 1:10 <sup>13</sup> pv/ml.)                                                        |
| RECUPERATION ET FILTRATION 0,2μm : | Récupération et pool des 2 Ultrafree pour chaque essai.<br>Filtration sur filtres Millex 0,2μ non stériles.<br>Stockage dans tubes en verre stériles.                                                                                               |
| ALIQUOTAGE : (1=0)                 | → 100µl dans tube Ependorff congelé à -26°C par essai.  → 20µl + 980µl tampon clhp anal. pour dosage.  → env. 900µl conservés à +4°C pour étude de stabilité.  → env. 100µl de la folution virale Y28 sortie chromato initiale est congelé à -26°C. |
| TEMOINS PBS/glycerol 10%:          | directement concentré à 1.10 <sup>13</sup> pv/ml, récupéré et aliquoté comme les autres essais.                                                                                                                                                     |

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# DOSAGES CLHP ANALYTIQUE.

| ESSAISTAMPONS                                         | TITRE pV/ml J=0          | APPARENCE DE<br>L'ECHANTILLON                                                  | TITRE OV/mil Je15                                                           | OBSERVATIONS CLHP du dusure [=15                                                 | TITRE pV/ml J=20 (*) Observations CLHP Apparence echantillan                          | TITRE pV(mt J=22 (*) Observations CLRP Apparence echanillon |
|-------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------|-----------------------------------------------------------------------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-------------------------------------------------------------|
| Tris 20mM                                             | 4,97.10 <sup>12</sup>    | normale à j=15                                                                 | non filue: 1,0.10 <sup>12</sup><br>filue 0,2μm: 9,1.10 <sup>11</sup>        | le retour pic adéno traîne<br>nbre plateaux:12000<br>avnétries:125 et 15         | non dosė<br>normale                                                                   |                                                             |
| Tempon 2<br>Tris+glycerol                             | 7,71. 10 <sup>17</sup>   | ingrale à j=15                                                                 | non filtre : 8,12,10 <sup>13</sup><br>filtre 0,2µm: 7,96,10 <sup>13</sup>   | pic symétrique                                                                   | non filtre : 7,88. 10 <sup>13</sup> pic symetrique normule                            | non filtre : 9,27,10 <sup>17</sup> pic symétrique nonnale   |
| <u>Tampon }</u><br>Tris+MgCl <sub>2</sub> +sucrose    | 6,29, 10 <sup>13</sup>   | opacification à j=12 <sup>4</sup><br>mais non précipité à j=15                 | non filtrė : 2,33,10 <sup>11</sup> .<br>filtrė 0,2µm: 2,09,10 <sup>11</sup> | monice du pic<br>asymétrique<br>hr nor plateanx:32000<br>asymétries:0,93 et 0.86 | non dosé<br>trouble mais non J                                                        |                                                             |
| Tampons 4 Tris+sucrose                                | 6,31. 10 <sup>12</sup>   | nonnale à j=15                                                                 | non filute : 5,83.10 <sup>12</sup><br>filute 0,2μm: 5,7.10 <sup>12</sup>    | pic synétrique                                                                   | non filtre:1,87.10 <sup>12</sup> ubre plateaux:14000 asynétries:1,28 et 1,42 norniale | non filtre :1,09,10 <sup>13</sup>                           |
| Tris+MgCl <sub>1</sub> +sucros <del>c+</del> mannitol | 5,84, 10 <sup>13</sup>   | normale à j=15                                                                 | non filue : 1,85.10 <sup>11</sup><br>filue 0,2μm: 1,47.10 <sup>12</sup>     | le retour pic adéno traîne<br>nbre plateaux:17000<br>avmètries:1.08 et 1.12      | non dosé<br>nomule                                                                    | -                                                           |
| Tris+NaCl+MgCl,+*ucrose+mannitol                      | 6,48. 10 <sup>17</sup>   | précipité à j=7                                                                | non filtre : non dosé<br>filtre 0,2 µm:                                     |                                                                                  | 1                                                                                     | 1.                                                          |
| <u>Tampon 7</u><br>Tristsucros <del>c</del> tiween    | 6,22. 10 <sup>12</sup>   | nonnale à j=15                                                                 | non filtre : 9,53.10 <sup>11</sup><br>filtre 0,2µm: 9,31.10 <sup>11</sup>   | sommet du pie arrondi<br>nbre plateaux:9000<br>asymétries:0-95 et (183           | bed non                                                                               |                                                             |
| Tempon 8 Tris+MgCl <sub>2</sub> +sucrose+tween        | 7,17. 10 <sup>17</sup>   | opacification à j=7<br>précipité le lendemain                                  | non filtre : non dose<br>filtre 0,2µm:                                      |                                                                                  |                                                                                       | 1                                                           |
| Tumpon 9 Borate+MgCl <sub>2</sub> +sucrose            | iion détecté             | virus retenu sur le filtre<br>solution trouble des le<br>changement de tampon; | non filue : non dose<br>filue 0,2µm:                                        |                                                                                  | 1                                                                                     | 1                                                           |
| Tames 10<br>Phosphate +glyctrol                       | 7,20. 10 <sup>12</sup>   | opweification d j=2<br>precipite le lendennain                                 | non filtre : non dose<br>filtre 0,2µm:                                      | 1                                                                                |                                                                                       |                                                             |
| Tampon 11 DPBS+NaCl+glycerol                          | . 5,37. 10 <sup>12</sup> | précipité à 1<1 jour                                                           | non filtre : non dose<br>filtre 0,2µm:                                      |                                                                                  |                                                                                       | 1                                                           |
| nota: pour le pic adéno étalon ->nbre plateaux: 32000 |                          | asymétries: 1,1 et 1,16                                                        |                                                                             |                                                                                  |                                                                                       |                                                             |

note: pour le pic addino étalon —nbre plateaux: 32000 /usymétries: 1,1 et 1,16 (\*) calcul des titres avec le nouvel étalon,441 Essais Tp2 et Tp4 retitrés à J=22 pour test bioactivité par M.Janicot

Service Con



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| SUJET: | ADONO VIRUS |  |  |
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## MISE EN PLACE DES ESSAIS DE STABILITE ADENOVIRUS DANS DIFFERENTES FORMUL

Echantillon de départ: 400ml fraction F3 (+10% glycérol) du DEMOBATCH 3 (CC16M-Ad5/CMV/P53/293), dosée à 3,6.10<sup>11</sup> pv/ml soit 1,44.10<sup>14</sup>pv pour 400ml.

#### Tampons étudiés (filtrés 0,22 µm):

- -Tampon A. Tris 20mM-pH8,4+10% glycerol
- -Tampon B: Tris 20mM-pH8,4+5% sucrose
- -Tampon C : Tris 20mM-pH8,4+10% glycérol+5% sucrose
- -Tampon D : Tris 20mM-pH8,4+5% glycérol+10% sucrose
- -Tampon E: Tris 20mM-pH8,4+10% glycerol+1mM MgCl<sub>2</sub>
- -Tampon F :Tris 20mM-pH8,4+ 10% glycerol+150mM NaCl+1mM MgCl<sub>2</sub>
- -Tampon G: Tris 20mM-pH8,4+5% glycérol
- -Tampon H: Tris 20mM-pH8,4+10% sucrose
- -Tampon 1 : Acétate d'ammonium 20mM-pH8+10% glycérol
- -Tampon J : Acétate d'ammonium 20mM-pH8+5% sucrose

#### Mise en place des essais :dans labo L3 de recherches/Bt Monod

- -1 tretape : concentration de l'échantillon en utilisant 16 Últrafree 15ml/30Kd membrane biomax (UFV2BTK40 Millipore), centrifugation à 1500tt/mn Premier passage, on amène le volume à 5ml, (il faut environ 30mn pour le passage de 5 ml) on recharge une deuxième fois les Ultrafree avec 10ml (on tourne à 1760tr/mn-500G) et on amène le volume total final à 105ml. on conserve 5ml pour électrophorèse 2D et on effectue un dosage HPLC (d1/10) on trouve 1,21.10<sup>12</sup>pv/ml soit 1,27.10<sup>14</sup>pv pour 105ml.
- etape : changement de tampon sur PD10 Pharmacia (4 PD10 par tampon, soit 4 fois 2,5ml du concentrat ou 1,21.1012 pv/tampon), on récupère 14ml.
- -3 me étape : on concentre les éluats PD10 sur un Ultrafree 15ml/30Kd (même réf. que étape 1) on amène le volume à <1 ml.
  - on récupère le concentrat et on volume à 1 ml avec le filtrat.
- \* étape : on fait subir à chaque échantillon une filtration stérilisante sur 🖢n filtre Millipore (Sterile Millex-GV 0,22μm) membrane PVDF , recupération dans un tube stérile.
- pour les échantillons TpA à E, aliquoter 14 tubes de 50µl, dans tubes stériles, pour les échantillons TpF à 1, il y a 15 aliquotes de 50µl. les titres se situent entre 9,8.10<sup>12</sup> et 1,08.10<sup>13</sup> pv/ml (voir cahier DOS-01 page 42)
- \* étape : les aliquotes de 50µl sont mis ce jour en stabilité à -20°C. les reliquats soit ~250 à 300µl sont conservés à 4°C.

ll est prevu un dosage pfu (labo D.Faucher) de chaque échantillon →1 tube de 50μl à -20°C

SUJE

# ENGLISH-LANGUAGE TRANSLATION OF EXHIBIT "A" (6 pages)

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**CEL 02051** 

#### FORMULATION TRIALS: STABILITY.

OBJECTIVE: Observe the stability, or possible precipitation, of the Y28 virus in

different formulations.

#### VIRAL MATERIAL STUDIED:

Y28 solution produced in a cell cube on an 8 mer scale by the J.F. Chaubard team, purified by ion exchange chromatography, and preserved in 20mM pH8 TRIS, 1mM MgCl<sub>2</sub>, 500mM NaCl, and 10% glycerol. The purified virus titrates 3.94.10<sup>11</sup> pv/ml.

#### PREPARATION OF THE DIFFERENT BUFFER SOLUTIONS USED:

#### 1. Stock solutions:

|   | STOCK SOLUTIONS:                      | PREPARATIONS:                                                                                                |
|---|---------------------------------------|--------------------------------------------------------------------------------------------------------------|
|   |                                       |                                                                                                              |
| Α | Tris / HCl pH 8.4 at 500mM            | 10.07g Tris base + 6.60g Tris/Hcl in 250ml water for injection (Tris base ref: T8524 and Tris HCL ref:T7149) |
| В | Sucrose at 50g/100ml                  | 250g sucrose in 500ml of water for injection.                                                                |
| С | NaCl 5M                               | Sigma - Aldrich ref. S150                                                                                    |
| D | MgCl <sub>2</sub> 1M                  | Sigma - Aldrich ref. M1028                                                                                   |
| E | Glycerol                              | Sigma - Aldrich ref. G5516                                                                                   |
| F | D-Mannitol                            | Sigma - Aldrich ref. M9647                                                                                   |
| G | Tween 20                              | Sigma - Aldrich ref. P8074                                                                                   |
| H | 100mM borate buffer solution pH 7.4   | 100mM boric acid + NaOH 0 <sub>2</sub> 1N                                                                    |
| 1 | 10mM phosphate buffer solution pH 7.4 | 130mg KH <sub>2</sub> PO <sub>4</sub> + 705mg K <sub>2</sub> HPO <sub>4</sub> in 500ml water for injection.  |

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#### 2. Formulations:

| ·        |           |               |                                       | STO                             | CK SOLUT                                  | IONS:                  |                           |                                       |               | ·                   |
|----------|-----------|---------------|---------------------------------------|---------------------------------|-------------------------------------------|------------------------|---------------------------|---------------------------------------|---------------|---------------------|
|          | Α         | В             | С                                     | D                               | Ε                                         | F                      | G                         | Н                                     | 1             | Water for injection |
| TRIAL:   | ·         |               |                                       |                                 |                                           |                        |                           |                                       |               |                     |
|          |           | <del></del> 1 |                                       |                                 | ı T                                       |                        |                           | ·                                     | · 1           | QS 500ml            |
| 11       | 20ml      |               |                                       | L                               | lk                                        |                        |                           |                                       |               |                     |
| 2        | 20ml      | [             |                                       |                                 | + 50ml                                    |                        |                           |                                       |               | QS 500ml            |
| <u> </u> |           |               |                                       |                                 |                                           |                        |                           | · · · · · · · · · · · · · · · · · · · | <del></del> 1 | 00 500ml            |
| 3        | 20ml      | 50ml          |                                       | 0,5ml                           | لــــــا                                  |                        | <u> </u>                  | لــــــا                              |               | QS 500ml            |
|          | 1 00 -1   | 50ml          |                                       | г :                             |                                           |                        | i -                       |                                       |               | QS 500ml            |
| 4        | 20ml      | ן טטווון      |                                       |                                 |                                           |                        | <u> </u>                  |                                       |               |                     |
| 5        | 20ml      | 50ml          |                                       | 0.5ml                           |                                           | 25g                    |                           |                                       |               | QS 500ml            |
|          |           |               | · · · · · · · · · · · · · · · · · · · |                                 | ·<br>                                     | 05                     | · ·                       | Τ                                     |               | QS 500ml            |
| 6        | 20ml      | 50ml          | 15ml                                  | 0.5ml                           |                                           | 25g                    |                           | <u> </u>                              |               | Q3 300m             |
| 7        | 20ml      | 50ml          | -                                     |                                 |                                           |                        | 0.5ml                     |                                       |               | QS 500ml            |
|          | 2011.     | 1             |                                       |                                 |                                           |                        |                           | · ·                                   | ·             |                     |
| 8        | 20ml      | 50ml          |                                       | 0.5ml                           | <u> </u>                                  | <u> </u>               | 0.5ml                     | <u> </u>                              |               | QS 500ml            |
|          |           |               |                                       | 0.5ml                           | т. —                                      | <u> </u>               | 1.                        | 50ml                                  | 1             | QS 500ml            |
| 9        | <u> </u>  | 50ml          |                                       | 0.5111                          | 1                                         | L                      |                           | 1 00                                  | <b>.</b>      |                     |
| 10       | T         |               |                                       | <u> </u>                        | + 50ml                                    |                        |                           |                                       | 500ml         |                     |
|          |           |               |                                       |                                 |                                           |                        |                           | tinal di                              | filtrotics    |                     |
| 11       |           | Vira          | al solution<br>Titer – 2              | obtained<br>88v10 <sup>11</sup> | in the seco                               | ona rinsin<br>PBS/1500 | g during the<br>mM NaCl/c | ilvcerol 1                            | 0%.           |                     |
| L        | <u>.i</u> |               | 11101 - 2                             |                                 | V 47 1111 111 111 111 111 111 111 111 111 |                        |                           |                                       |               |                     |

### 3. Summary of the formulations studied:

See the following tables.

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Y28 CELL CUBE 8MER

FORMULATION TRIALS

|              |                           |                                   |   |   |    |          |     |     | _ | _ | _     | _ | _ | _          | _ | 1 |
|--------------|---------------------------|-----------------------------------|---|---|----|----------|-----|-----|---|---|-------|---|---|------------|---|---|
| 10mM pH/.4   | phosphate buffer solution |                                   |   |   |    |          |     |     |   |   |       |   | 4 | +          |   |   |
| 10 mM horate | pH7.4 buffer solution     |                                   |   |   |    |          |     |     |   |   |       | + |   |            |   |   |
|              | DPBS                      |                                   |   |   |    |          |     | 7.0 |   | * |       |   |   | , y<br>, y | + | - |
|              | Glycerol<br>10%           |                                   |   | + |    |          |     |     |   |   |       |   |   | +          | 4 | ۲ |
|              | Mannitol<br>5%            | <b>T</b>                          |   |   |    |          | +   | -   | + |   |       |   |   |            |   |   |
|              | Tween20<br>0.1%           |                                   |   |   |    |          |     |     |   | + | +     |   |   |            |   |   |
|              | Sucrose 5%                |                                   |   |   | +  | +        | +   |     | + | + | +     |   | + |            |   |   |
|              | MaCl <sub>2</sub>         |                                   |   |   | +  |          | +   | -   | + |   | +     | - | + |            | 1 |   |
|              | NaCl<br>150mM             | COP 20 ST COM COM PER CONTROL COM |   |   |    |          | 4   |     | + |   |       |   |   |            |   | + |
|              | Tris<br>20mM              | pH 8.4                            | + | + | +  | +        |     | +   | + | + |       | + |   |            |   |   |
|              | TRIAL                     |                                   | - | 2 | 60 | <b>V</b> | - 1 | n   | 9 | 7 | .   0 | 0 | 6 | Ş          | 2 | - |

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#### MATERIALS USED:

- ightarrow 10 PD 10 for diafiltration balanced with 5 x 5ml of the buffer solution studied.
- ightarrow 15ml Ultrafree with 100 Kd Biomax (Millipore) membrane (2x for each trial).
- $\rightarrow$  Centrifuge set at 1500 rev/min.

#### IMPLEMENTATION:

| OPERATIONS:                    | FOR EACH TRIAL:                                                       |
|--------------------------------|-----------------------------------------------------------------------|
| DIAFILTRATION:                 | 10 PD10 x 2.5ml of Y28 viral solution at 3.94.10 <sup>11</sup> pv/ml. |
| DIA ETTATO:                    | Elution by 10PD10 x 3.5ml of the buffer solution studied.             |
| CONCENTRATION:                 | 15ml 100Kd 2 Ultrafree filled to 15ml and then refilled               |
| 001102111111                   | with 2.5 diafiltrated viral solution.                                 |
|                                | 17.5ml concentrated at 500µl (x2).                                    |
|                                | (or a concentration at ≈ 1.10 <sup>13</sup> pv/ml.)                   |
|                                |                                                                       |
| RECOVERY AND FILTRATION 0.2μm: | Recovery and pooling of the 2 Ultrafree for each trial.               |
| NEOOVERT AND THE VIEW          | Filtration using unsterilized 0.2µ Millex filters.                    |
|                                | Storage in sterilized glass tubes.                                    |
|                                |                                                                       |
| ALIQUOTING: (t=0)              | → 100µl in Ependorff tube frozen at -26°C. → *                        |
|                                | → 20µl + 980µl anal. HPCL buffer solution for dosing.                 |
|                                | → About 900µl stored at +4°C to study stability.                      |
|                                | → About 100µl of the initial chromate emerging Y28 viral              |
|                                | is frozen at -26°C.                                                   |
| 0                              |                                                                       |
| 10% glycerol/PBS Samples:      | Frozen directly at 1.10 <sup>13</sup> pv/ml, recovered and aliquoted  |
|                                | in the same way as the other trials.                                  |

TRIAL NO.

ANALYTICAL HPLC MEASUREMENTS:

| Eurite 2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | TRIAL S/BUFFERS                                           | TITER pV/ml J=0        | SAMPLE APPEARS                                                                | TITER pV/ml<br>day=15                                                         | OBSERVATIONS HPLC of the dosage, dav=15                                                  | TITER pV/ml day=20(*) HPLC Observations Sample appears                                     | TITER pV/ml day=22(*) HPLC Observations Sample appears                                    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|------------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| 6.29 10² copacification at day=15 filtered 2µm: peak number at day=15 filtered 2µm: peak number at day=15 filtered 2µm: peak number at day=15 filtered 2µm: peak aymmetrical peak number at day=15 filtered 0,2µm: peak number at day=16 filtered 0,2µm: peak number                                                                                                                                                                 | Buffer 1<br>Tris 20mM                                     | 4.97.10 <sup>12</sup>  | normal at day=15                                                              | unfiltered: 1.0.10 <sup>12</sup><br>filtered 0.2µm:<br>9.1.10 <sup>1</sup>    | The adeno retum peak trails plate number: 12,000 asymmetries: 1.25 and 1.50              | not tested<br>normal                                                                       | ŀ                                                                                         |
| 6.29. 10 <sup>12</sup> opecification at day=12 <sup>2</sup> unfiltered: 2.33 10 <sup>11</sup> the peak asymmetrical rise of clouding but not 1 to plate number: 3.000  6.31. 10 <sup>12</sup> normal at day=15 intered 0.2 μm: plate number: 3.000  6.34. 10 <sup>12</sup> normal at day=15 intered 0.2 μm: plate number: 3.000  6.34. 10 <sup>12</sup> normal at day=15 intered 0.2 μm: plate number: 1.000  8.48. 10 <sup>12</sup> normal at day=7 unfiltered: 1.83.10 <sup>11</sup> plate number: 1.000  8.48. 10 <sup>12</sup> normal at day=7 intered 0.2 μm: plate number: 1.000  8.49. 10 <sup>12</sup> normal at day=7 intered 0.2 μm: asymmetries: 0.6 and 1.42 (normal)  9.31.10 <sup>11</sup> asymmetries: 0.6 and 1.12  1.47.10 <sup>12</sup> precipitated the next of illered 0.2 μm: asymmetries: 0.65 and 0.88  1.47. 10 <sup>12</sup> normal at day=7 illered 0.2 μm: asymmetries: 0.65 and 0.88  1.47. 10 <sup>12</sup> normal at day=7 illered 0.2 μm: asymmetries: 0.65 and 0.88  1.47. 10 <sup>12</sup> normal at day=7 illered 0.2 μm: asymmetries: 0.65 and 0.88  1.47. 10 <sup>12</sup> normal at day=7 illered 0.2 μm: day normal at day=7 illered 0.2 μm: day normal at day=2 illered 0.2 μm: day=2 illered 0.2 μm: day normal at day=2 illered 0.2 μm: day=2 illered 0.2 μm: day=2 illered 0.2 μm: day=2 illered 0.2 μm: day=2 i | Buffer 2<br>Tris*glycerol                                 | 7.71. 10 <sup>12</sup> | normal at day=15                                                              | unfiltered: 8.12.10 <sup>12</sup><br>filtered 0.2µm:<br>7.96.10 <sup>12</sup> | symmetrical peak                                                                         | unfiltered: 7.88.10 <sup>12</sup><br>normal symmetrical<br>peak                            | unfiltered: 9.27.10 <sup>12</sup><br>normal symmetrical<br>peak                           |
| 6.31. 10 <sup>12</sup> normal at day=15 unfiltered: 5.83.10 <sup>12</sup> symmetrical peak plate number: 1.80 plate number: 1.80 plate number: 1.80 and 1.42 (normal) illered 0.2µm: plate number: 1.7000 plate number: 1.000 plate number: 1.28 and 1.42 (normal) illered 0.2µm: plate number: 1.000 plate number: 1.000 plate number: 1.000 asymmetries: 1.08 and 1.42 (normal) illered 0.2µm: plate number: 1.000 asymmetries: 1.08 and 1.42 (normal) and 1.47.10 <sup>12</sup> precipitated at day=15 filtered 0.2µm: asymmetries: 0.95 and 0.83 and 1.12 asymmetries: 0.95 and 0.83 and 0.2µm: day.  C.17. 10 <sup>12</sup> opacification at day=7 filtered 0.2µm: filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>12</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>13</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>14</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>15</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>16</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>17</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>18</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  C.17. 10 <sup>18</sup> opacification at day=2 filtered 0.2µm: day.  C.17. 10 <sup>18</sup> opacification at day=2 filtered 0.2µm: day.  C.17. 10 <sup>18</sup> opacification at day=2 filtered 0.2µm: day.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Buffer 3<br>Fris+MgCl <sub>3</sub> + sucrose              | 6.29. 10 <sup>12</sup> | opacification at day=12³ but not precipitated at day=15                       | unfiltered: 2.33.10 <sup>11</sup><br>filtered 0.2µm:<br>2.09.10 <sup>11</sup> | asymmetrical rise of<br>the peak<br>plate number: 32,000<br>asymmetries: 0.93 at<br>0.86 | not tested<br>clouding but not i                                                           | l                                                                                         |
| 5.84. 10 <sup>12</sup> normal at day=15 (iltered: 1.85.10 <sup>12</sup> trails (iltered: 0.2µm: plate number: 17,000 asymmetries: 1.08 and 1.12  6.48. 10 <sup>12</sup> precipitated at day=7 (iltered: 0.2µm: plate number: 17,000 asymmetries: 0.95 and 1.12 (iltered: 0.2µm: plate number: 9,000 plat                                                                                                                                   | Buffer 4<br>Tris+sucrose                                  | 6.31. 10 <sup>12</sup> | normal at day=15                                                              | unfiltered: 5.83.10 <sup>12</sup><br>filtered 0.2µm:<br>5.7.10 <sup>12</sup>  | symmetrical peak                                                                         | unfiltered: 1.87.10 <sup>12</sup> plate number: 14,000 asymmetries: 1.28 and 1.42 (normal) | unfiltered: 1.09.10 <sup>12</sup> plate number: 4,000 asymmetries: 0.87 and 0.68 (normal) |
| 6.48. 10 <sup>12</sup> precipitated at day=7 unfiltered: not tested — filtered 0.2µm:  6.22. 10 <sup>12</sup> normal at day=15 filtered 0.2µm:  9.31.10 <sup>11</sup> asymmetries: 0.95 and 0.83  7.17. 10 <sup>12</sup> opacification at day=7 unfiltered: not tested precipitated the next day.  Undetected virus held on the filter solution is changed.  7.20. 10 <sup>12</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  7.20. 10 <sup>12</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day.  7.20. 10 <sup>12</sup> precipitated the next filtered 0.2µm: filtered 0.2µm: filtered 0.2µm: filtered 0.2µm: filtered 0.2µm:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Buffer 5<br>MgCl <sub>2</sub> +sucrose+mannitol           | 5.84. 10 <sup>12</sup> | normal at day=15                                                              | unfiltered: 1.85.10 <sup>12</sup><br>filtered 0.2µm:<br>1.47.10 <sup>12</sup> | The adeno retum peak trails plate number: 17,000 asymmetries: 1.08 and 1.12              | not tested<br>normal                                                                       | 1                                                                                         |
| 6.22. 10 <sup>12</sup> normal at day=15 filtered 0.2µm: asymmetries: 0.95 and 0.83  7.17. 10 <sup>12</sup> opacification at day=7 day.  Undetected virus held on the filter solution is changed.  7.20. 10 <sup>12</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: changed.  7.20. 10 <sup>12</sup> opacification at day=2 precipitated the next day  7.20. 10 <sup>12</sup> opacification at day=2 filtered 0.2µm: filtered 0.2µm: day filtered at < 1 day filtered 0.2µm: day filtered at < 1 day filtered 0.2µm: day filtered 0.2µm: day filtered 0.2µm: day filtered 0.2µm:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Buffer 6<br>+NaCl+MgCl <sub>2</sub> +sucrose+<br>mannitol | 6.48. 10 <sup>12</sup> | precipitated at day=7                                                         | unfiltered: not tested<br>filtered 0.2µm:                                     | ı                                                                                        | -                                                                                          | 1                                                                                         |
| 7.17. 10 <sup>12</sup> opacification at day=7 precipitated the next day.  Undetected virus held on the filter solution clouds once the buffer solution is changed.  7.20. 10 <sup>12</sup> opacification at day=2 precipitated the next day  5.37. 10 <sup>12</sup> precipitated at < 1 day                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Buffer 7<br>Tris+sucrose+Tween                            | 6.22. 10 <sup>12</sup> | normal at day=15                                                              | unfiltered: 9.53.10 <sup>11</sup><br>filtered 0.2µm:<br>9.31.10 <sup>11</sup> | rounded peak top<br>plate number: 9,000<br>asymmetries: 0.95 and<br>0.83                 | not tested<br>normal                                                                       | 1                                                                                         |
| Undetected virus held on the filter solution clouds once the buffer solution is changed.  7.20. 10 <sup>12</sup> opacification at day=2 precipitated the next day  5.37. 10 <sup>12</sup> precipitated at < 1 day                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Buffer 8<br>+MgCl <sub>2</sub> +sucrose+Tween             | 7.17.10 <sup>12</sup>  | opacification at day=7 precipitated the next day.                             | unfiltered: not tested<br>filtered 0.2µm:                                     | 1                                                                                        | l' .                                                                                       |                                                                                           |
| 7.20. 10 <sup>12</sup> opacification at day=2 precipitated the next day day 5.37. 10 <sup>12</sup> precipitated at < 1 day                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Buffer 9<br>orate+MgCl <sub>2</sub> +sucrose              | Undetected             | virus held on the filter solution clouds once the buffer solution is changed. | unfiltered: not tested<br>filtered 0.2µm:                                     | 1                                                                                        | 1                                                                                          | 1                                                                                         |
| 5.37. 10 <sup>12</sup> precipitated at < 1 day                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Buffer 10<br>Phosphate + glycerol                         | 7.20. 10 <sup>12</sup> | opacification at day=2<br>precipitated the next<br>day                        | unfiltered: not tested<br>filtered 0.2µm:                                     | I                                                                                        | _                                                                                          | 1'                                                                                        |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Buffer 11<br>DPBS+NaCl+glycerol                           | 5.37. 10 <sup>12</sup> | precipitated at < 1 day                                                       | unfiltered: not tested<br>filtered 0.2µm:                                     | I                                                                                        | _                                                                                          | <b>1</b>                                                                                  |

Note: for the adeno return peak measurement standard → plate n (\*) computation of titers with the new measurement standard: 141

A-5 Page 5 of 5

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SUBJECT: ADENOVIRUS

#### CONDUCTING ADENOVIRUS STABILITY TRIALS IN DIFFERENT FORMULATIONS

Starting sample: 400ml fraction F3 (+10% glycerol) of DEMOBATCH 3 (CC16M-Ad5/CMV/P53/293) dosed at 3.6.10<sup>11</sup> pv/ml or 1.44.10<sup>14</sup>pv per 400ml.

#### Buffer solutions studied (0.22µm filtered):

- -Buffer solution A: Tris 20mM-pH8.4+10% glycerol
- -Buffer solution B: Tris 20mM-pH8 4+5% sucrose
- -Buffer solution C: Tris 20mM-pH8.4+10% glycerol+5% sucrose
- -Buffer solution D: Tris 20mM-pH8.4+5% glycerol+10% sucrose
- -Buffer solution E: Tris 20mM-pH8.4+10% glycerol+1mM MgCl<sub>2</sub>
- -Buffer solution F: Tris 20mM-pH8.4+10% glycerol+150mM NaCl+1mM MgCl<sub>2</sub>
- -Buffer solution G: Tris 20mM-pH8.4+5% glycerol
- -Buffer solution H: Tris 20mM-pH8.4+10% sucrose
- -Buffer solution 1: ammonium acetate 20mM-pH8+10% glycerol
- -Buffer solution 1: ammonium acetate 20mM-pH8+5% sucrose

#### Carrying Out the Trials: At Research Lab L3/Bt Monod.

1<sup>st</sup> Step: Concentrating the sample by using 15ml/30Kd 16 Ultrafee biomax membrane (UFV2BTK40 Millipore), centrifuged at 1500rev/min. First run, volume brought to 5ml (5ml run requires @30 mins). The Ultrafree is filled a second time with 10ml (turning occurs at 1760 rv/min.-500G). The final total volume is brought to 105ml. 5ml is stored for 2D electrophoresis and HPLC (dl/10) measurement occurs. One then finds 1.21.10<sup>12</sup>pv/ml, or 1.27.10<sup>14</sup> pv per 105ml.

2<sup>nd</sup> Step: Changing over the sample to PD10 Pharmacia (4 PD10 by buffer solution, i.e., 4 x 2.5ml of the concentrate or 1.21.10<sup>13</sup>pv/buffer solution), 14ml are recovered.

3<sup>rd</sup> Step: The PD10 eluates are concentrated on a 15ml/30Kd Ultrafree (same ref. as Step 1) and the volume is brought to <1ml. The concentrate is recovered and the volume is increased to 1ml with filtrate.

4<sup>th</sup> Step: Each sample undergoes a sterilizing filtration on a Millipore film (Sterile Millex-GV 0.22μm) membrane (PVDF). Collected in a sterile tube.

 $5^{th}$  Step: On each 1ml sample after filtration  $\rightarrow$ HPLC (d1/50). For samples TpA to E, aliquot 14 tubes of 50µl in sterile tubes. For samples TpF to J, there are 15 aliquots of 50µl. The titers are located between 9.8.10<sup>12</sup> and 1.08.10<sup>13</sup> pv/ml (see Manual DOS-01 page 42).

 $6^{th}$  Step: The 50 $\mu$ m aliquots are used while stable at -20 $^{\circ}$ C. The carry-over, i.e., 250 to 300 $\mu$ l, is stored at  $4^{\circ}$ C.

A PFU (D. Faucher Lab) measurement of each sample is provided →1 tube of 50μl at -20°C.

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